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CYCLOSPORIN A INHIBITS BIOLOGICAL EFFECTS OF TUMOR PROMOTING PHORBOL ESTERS

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The antilymphocytic and antiphlogistic agent cyclosporin A (CsA) inhibits in vivo various effects induced by the tumor promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA). These include the edema of the mouse ear, the alkaline phosphatase (AP) activity and the ornithine decarboxylase (ODC) activity in mouse epidermis as well as the generation of a specific arachidonic acid (AA) metabolite in mouse epidermis. AA metabolism in an epidermal cell-free system of mouse epidermis was not suppressed by CsA. According to thin layer chromatography the TPA-induced and as yet unidentified AA metabolite exhibits a polarity between that of 5-HETE and 12-/15-HETE. Studies with inhibitors indicate it to be a lipoxygenase product.

CsA is an immune suppressant with antilymphocytic activity (1). The immunobiology of CsA has been reviewed recently by Thomson (2). Probably most important for the immunosuppressive capacity of CsA is its ability to inhibit the production of lymphokines, e.g. interleukin 1 and 2, as well as to inhibit the responsiveness of T cells to these lymphokines (3-5). The mode of this inhibitory action is not yet known. Recently, however, CsA receptors were demonstrated on human lymphocytes (6) and it was reported that CsA inhibits lymphocyte activation by interfering with the early changes in the phospholipid metabolism of the lymphocyte plasma membrane (7). A further property of CsA is its antiphlogistic activity (1). It is not known whether this activity of CsA is related to its antilymphocytic activity or whether both activities are entirely independent properites of the compound. Very recently it was shown that the suppression of tissue levels of prostaglandins by CsA might be due to an inhibition of phospholipase A_2 (8).

Tumor promotion is a complex process in which a visible tumor develops presumably from a single cell that has been initiated by a carcinogen. The

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most thoroughly studied system in this respect is tumor promotion by phorbol esters in mouse epidermis (9). Although a still increasing number of effects of phorbol esters in vivo and in vitro has been described, the mechanism of tumor promotion is unknown as yet. Inhibitors of tumor promotion and of various effects of phorbol esters should be valuable tools for the elucidation of this mechanism. Here we report on the TPA-induced generation of an AA metabolite and on the inhibition by CsA of this and other effects of tumor promoting phorbol esters on mouse epidermis in vivo.

MATERIAL AND METHODS

Materials:

[^{14}C(U)]-Arachidonic acid (spec.act.390 mCi/mmole; [^{14}C]AA) [^{3}H(N)]-5-D-hydroxy-6,8,11,14-eicosatetraenoic acid ([^{3}H]-5-HETE) (spec.act. 30-60 Ci/mmole), [^{3}H(N)]-12-L-hydroxy-5,8,10,14-eicosatetraenoic acid ([^{3}H]-12-HETE) (spec.act.30-60 Ci/nmole) and [^{3}H(N)]-15-L-hydroxy-5,8,11,13-eicosatetraenoic acid ([^{3}H]-15-HETE) (spec.act. 30-100 Ci/mmole) were from New England Nuclear (Waltham,MA). Cyclosporin A was a generous gift of Sandoz, Basel, Switzerland. TPA was kindly supplied by Prof. Dr. E. Hecker, German Cancer Research Center, Heidelberg, F.R.G.

Animals: Female NMRI mice (age 7 to 8 weeks) were used in all experiments.

Edema induction and measurement: as described previously (10).

Alkaline phosphatase (AP) assay: as described previously (11).

Ornithine decarboxylase (ODC) assay: as described by O'Brien et al. (12).

Determination of protein: was carried out according to Lowry et al. (13).

Generation and analysis of arachidonic acid (AA) metabolites:

Epidermal specimens were prepared from frozen back skin of mice and homogenized as described previously (11). The homogenate was centrifuged at 8,000 x g for 1 min. One ml of the supernatant (epidermal cell-free system; 800 µg protein/ml) was incubated with 10 µl of a 1:1 mixture of [^{14}C]AA and unlabeled AA at 37°C for 45 min. After cooling in ice, 3 ml of chloroform/methanol (19:1) were added. After extensive mixing, the phases were separated by centrifugation at 10,000 x g for 5 min. 2.5 ml of the chloroform/methanol phase were evaporated to dryness and redissolved in 30 µl chloroform/methanol (2:1). A 25 µl aliquot was analyzed by thin layer chromatography on silica 60 (Merck/Darmstadt) using ether/hexane/acetic acid (60:40:1) as developing solvent. The chromatographic analysis was recorded by a radiochromatogram scanner (Berthold).

RESULTS AND DISCUSSION

The inhibition of various TPA effects in vivo by CsA is shown in Table 1. The edema of the mouse ear, which was found to be maximal 6 h after the

Table 1: Inhibition of various TPA effects by CsA in vivo

TPA-effect	Dose of CsA	Inhibition (%)
Edema (mouse ear)[a]		
	25 µg	31
	50 µg	75
	100 µg	91
	250 µg	87
AP activity (mouse skin)[b]		
	1.5 mg	60
	2.5 mg	100
ODC activity (mouse skin)[c]		
	2.0 mg	92
Arachidonic acid metabolite (mouse skin)[d]		
	2.5 mg	100

A single dose of CsA, as indicated, was applied together with TPA.

[a] The edema was measured 6 h after TPA treatment. Each value is the mean of 4 determinations. With 1 nmole TPA alone the weight of the ear plug increased from 10.45 to 21.54 mg (difference: 11.09 mg = 0% inhibition). For the procedure see ref. 10.

[b] AP activity was measured 22 h after TPA treatment. Each value is the mean of 6 determinations. With 20 nmole TPA alone the absorbance at 405 nm increased from 0.77 to 8.80/mg protein (difference: 8.03/mg protein = 0% inhibition). For the procedure see ref. 11.

[c] ODC activity was measured 4 h after TPA treatment. The value is the mean of 2 determinations. With 20 nmole TPA alone the radioactivity increased from 80 to 4537 cpm (difference: 4457 cpm = 0% inhibition). For the procedure see ref. 12.

[d] see Figure 1

application of 1 nmole TPA (10), was suppressed to about 90% by 100 µg of CsA. Besides the immunosuppressive properties of CsA, an antiphlogistic activity in chronic inflammation has already been demonstrated (1). Based on our recent findings, we proposed that all inhibitors of the TPA-induced edema are also inhibitors of skin tumor promotion (10). This hypothesis is presently tested with CsA. The other effects of TPA were determined in the back skin of mice. Because of the larger area for the topical application, 20 nmole of TPA and up to 2.5 mg of CsA were used. Recently, we found that AP activity in mouse epidermis was increased more than 10-fold 22 h after a single treatment of the skin with tumor promoting phorbol esters (11). This effect was completely abolished if 2.5 mg CsA were applied together with

TPA. As discussed previously (11), several data indicated that the TPA-induced AP activity in mouse epidermis arose from leaking endothelial cells that had been damaged by TPA. If this holds true, CsA should be able to increase capillary resistance. To our knowledge, however, a stabilizing effect of CsA on the microvasculature has not been described as yet. Another wellknown TPA effect, the increase in epidermal ODC activity (12), was also suppressed very efficiently by CsA. Finally, CsA inhibited completely the TPA-induced generation of an AA metabolite in mouse epidermis *in vivo* (Fig. 1 and Table 1). The as yet unidentified metabolite migrated on thin layer chromatography between 5- and 12-/15-HETE (Figure 1). To our

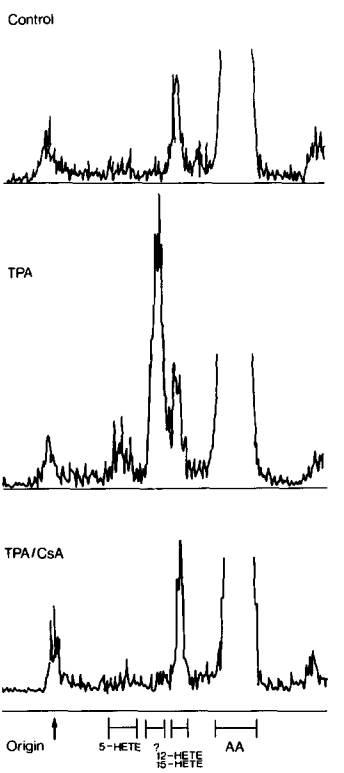

Figure 1: Radiochromatogram of AA metabolites generated in mouse epidermis in vitro.

Two hundred µl acetone alone (control), 20 nmole TPA in acetone or 20 nmole TPA/2.5 mg CsA in acetone were applied to the shaved back skin of 2 mice each. 24 hours later the mice were sacrificed. Epidermal specimens were prepared from the back skin, homogenized and used for the generation of AA metabolites in vitro as described in Methods. Extraction of metabolites, separation by thin layer chromatography and analysis of the radiochromatogram were performed as described in Methods. [^{3}H]-5-HETE, [^{3}H]-12-HETE and [^{3}H]-15-HETE served as standards. The profiles were reproduced in 5 independent experiments.

knowledge the induction by TPA of such a metabolite has not been reported previously. Lysed leucocytes from mouse blood did not produce this metabolite *in vitro*, even though they metabolized AA to HETEs efficiently (data not shown). Thus the metabolite was not generated by "ordinary" leucocytes invading the epidermis after TPA treatment. Nevertheless, we cannot exclude that *in vivo* TPA caused leucocytes to produce this metabolite and that it was found in our assay due to a contamination with such "TPA-activated" leucocytes. CsA was unable to inhibit AA metabolism in the epidermal cell-free system. The *in vitro* metabolism of AA in this system including the generation of the TPA-induced metabolite was suppressed completely, however, by the lipoxygenase inhibitors quercetin, nordihydroguaiaretic acid and sesamol, but not by the cyclooxygenase inhibitor indomethacin (data not shown). This indicates in the first place that the TPA-induced metabolite was generated along the lipoxygenase rather than the cyclooxygenase pathway and, secondly, that CsA did not simply act as a lipoxygenase inhibitor. TPA-stimulated protein kinase C (PKC), which is thought to play a central role in tumor promotion (14), was not suppressed by CsA in the epidermal cell-free system. Recently, we reported on the stimulation of PKC by TPA and on its suppression by various inhibitors in an epidermal cell-free system (15). For instance, 2×10^{-5}M phloretin caused a 50% inhibition of the TPA-stimulated PKC-activity. CsA was ineffective in this respect even at 5×10^{-4}M. Thus it is not likely that CsA inhibited the TPA effects *in vivo* by directly inhibiting the TPA-stimulated PKC. This does not exclude, however, an indirect inhibition of PKC by CsA *in vivo*. Furthermore, it is possible that a process following the stimulation of PKC by TPA was suppressed by CsA *in vivo*. Taken together, the results indicate that the immunosuppressive agent CsA is a very potent inhibitor of several TPA effects. One possibility is that there is a common TPA-induced process leading to these various effects and that this process can be inhibited by CsA. It remains an open question whether the immunosuppressive activity of CsA is based also on the inhibition of this as yet unknown process.

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